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SHORT COMMUNICATION

$p19^{Arf}$ is required for the cellular response to chronic DNA damage

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The p53 tumor suppressor is a stress sensor, driving cell cycle arrest or apoptosis in response to DNA damage or oncogenic signals. p53 activation by oncogenic signals relies on the $p19^{Arf}$ tumor suppressor, while p53 activation downstream of acute DNA damage is reported to be $p19^{Arf}$-independent. Accordingly, $p19^{Arf}$-deficient mouse embryo fibroblasts (MEFs) arrest in response to acute DNA damage. However, $p19^{Arf}$ is required for replicative senescence, a condition associated with an activated DNA damage response, as $p19^{Arf}-/-$ MEFs do not senesce after serial passage. A possible explanation for these seemingly disparate roles for $p19^{Arf}$ is that acute and chronic DNA damage responses are mechanistically distinct. Replicative senescence may result from chronic, low-dose DNA damage responses in which $p19^{Arf}$ has a specific role. We therefore examined the role of $p19^{Arf}$ in cellular responses to chronic, low-dose DNA-damaging agent treatment by maintaining MEFs in low oxygen and administering 0.5 G y γ-irradiation daily or 150 μM hydroxyurea, a replication stress inducer. In contrast to their response to acute DNA damage, $p19^{Arf}-/-$ MEFs exposed to chronic DNA damage do not senesce, revealing a selective role for $p19^{Arf}$ in senescence upon low-level, chronic DNA damage. We show further that p53 pathway activation in $p19^{Arf}-/-$ MEFs exposed to chronic DNA damage is attenuated relative to wild-type MEFs, suggesting a role for $p19^{Arf}$ in fine-tuning p53 activity. However, combined Nutlin3a and chronic DNA-damaging agent treatment is insufficient to promote senescence in $p19^{Arf}-/-$ MEFs, suggesting that the role of $p19^{Arf}$ in the chronic DNA damage response may be partially p53-independent. These data suggest the importance of $p19^{Arf}$ for the cellular response to the low-level DNA damage incurred in culture or upon oncogene expression, providing new insight into how $p19^{Arf}$ serves as a tumor suppressor. Moreover, our study helps reconcile reports suggesting crucial roles for both $p19^{Arf}$ and DNA damage-signaling pathways in tumor suppression.

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INTRODUCTION

It has long been appreciated that mammalian cells undergo a permanent growth arrest response, termed cellular senescence, upon continued propagation in culture. In addition, cells can be driven to undergo an accelerated senescence response upon expression of select oncogenes. Both of these responses provide safeguard mechanisms against neoplasia, by blocking proliferation either in suboptimal growth conditions or in response to inappropriate proliferative signals. The importance of senescence in tumor suppression is underscored by the fact that both senescence responses are critically dependent on the p53 tumor suppressor, as well as its positive regulator, the Arf tumor suppressor, which acts to stabilize p53 through sequestration of the Mdm-2 ubiquitin ligase. Both $p19^{Arf}$- and p53-deficient mouse fibroblasts are immortal, failing to undergo senescence upon passaging in culture or in response to expression of oncogenes such as activated Ras.[1]

Although one of the major cues that signals senescence with increasing passage of human cells is the attrition of telomeres, senescence-activating signals in mouse cells, which typically have long telomeres >20 kb, have remained less clear.[2,3] Senescence in mouse fibroblast cells has been correlated with high oxygen tension, as maintenance of cultures in low oxygen inhibits activation of the senescence program, either with increasing passage or with expression of oncogenes such as activated Ras.[4–7] Oxidative stress in fact not only contributes to senescence in mouse cells but also in human cells, as treatment of activated Ras-expressing human fibroblasts with scavengers of reactive oxygen species, such as *N*-acetyl cysteine, inhibits senescence,[6] implicating reactive oxygen species in the senescence response. As one of the major consequences of reactive oxygen species accumulation is the induction of DNA strand breaks, the senescence induced under high oxygen tension is likely at least partially triggered by DNA damage.[7,8] This notion has been supported by the observation that oncogene-induced senescence relies on components of the DNA damage-signaling cascade, including Atm and Chk2.[9–11]

Although the idea that a DNA damage signal induces senescence is consistent with the known requirement of p53, a sensor of DNA damage, this model does not account for the participation of $p19^{Arf}$, which has been reported to be dispensable for the response of cells to DNA damage.[1,12–15] One possible explanation for this discrepancy is that the studies examining the role of $p19^{Arf}$ and p53 in the DNA damage response have typically been done *in vitro* through exposure of cells to a single, high dose of a particular genotoxin (such as 5–20 Gy γ-irradiation), which elicits a rapid cellular response, typically within 24 h.[16–18]

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In contrast, the DNA damage driving senescence in cultured cells is chronic, low-level damage. Likewise, the DNA damage encountered during tumor development is not due to a single, acute genotoxic insult but rather continual, low-level genomic damage caused by unscheduled DNA replication and consequent replication fork collapse, telomere attrition, and increased reactive oxygen species levels resulting from enhanced metabolic activity or hypoxia/reperfusion.[6,19–24] Thus, examining the mechanisms underlying responses to lower levels of chronic DNA damage, rather than a high acute dose, will likely provide a more accurate picture of how p53 acts to suppress cancer *in vivo*.

Here, we leverage a widely utilized mouse embryonic fibroblast (MEF) model system to examine the roles of p53 and p19^{Arf} in the cellular responses to acute high-dose and chronic low-dose DNA damage. To allow controlled exposure to exogenous DNA damage, we grow cells in low-oxygen conditions to mitigate the effects of culture shock. Interestingly, we find that while both wild-type and *p19^{Arf}*−/− fibroblasts undergo cell cycle arrest upon exposure to a single high dose of a DNA-damaging agent, wild-type cells also undergo cell cycle arrest upon chronic treatment with a low-dose DNA-damaging agent, but *p19^{Arf}*−/− fibroblasts fail to do so. These findings suggest that p19^{Arf} is critical for the response to the low-level DNA damage incurred under culture conditions or upon oncogene expression and provide new insight into replicative senescence. Importantly, our findings also help reconcile the contradiction that various genetic studies have shown that the response of p53 to acute DNA damage is dispensable for tumor suppression, while other studies have shown that DNA damage signals are observed in early tumors and suggested that these signals are important for p53-mediated tumor suppression.[20,21,25,26] Our observations suggest a mechanistic difference in the p53-dependent pathways leading to senescence/tumor suppression in response to acute and chronic DNA damage signals, in terms of the contribution of p19^{Arf}. Our findings thus refine our understanding of p53-mediated tumor suppression by indicating that while p53's ability to respond acute DNA damage is dispensable for tumor suppression, its ability to drive responses to chronic DNA damage may be crucial, providing significant new insight into both senescence and tumor suppression.

RESULTS AND DISCUSSION

Establishing assays for acute and chronic DNA damage responses

To study the cellular response to chronic, low-dose DNA damage, it was first necessary to develop assays for chronic and acute DNA damage responses. We defined an acute DNA-damaging agent as one where a single, high-dose treatment triggers rapid cell cycle arrest and a low-dose DNA-damaging agent as one where a single treatment fails to cause a proliferative arrest. Treatment with the DNA double-strand break inducer doxorubicin or 12 Gy γ-irradiation allows examination of acute DNA damage responses, as wild-type MEFs respond to these treatments by undergoing proliferative arrest 24 h after treatment (Figure 1a). In contrast, exposure to 0.5 Gy γ-irradiation, or to 150 μM hydroxyurea (HU)—a ribonucleotide reductase inhibitor and inducer of replication stress, a type of stress encountered by developing cancer cells[11,27]—does not induce cell cycle arrest, as BrdU incorporation is similar to untreated cells 24 h after treatment (Figure 1a). To correlate arrest responses with p53 activity, we assessed p53 stabilization and transcriptional activity under these conditions. Although doxorubicin induced robust p53 protein stabilization after 8 h, 12 Gy γ-irradiation produced more modest p53 stabilization (Figure 1b). Both treatments, however, induced expression of the p53 target genes *p21* and *Mdm2* 2 h after treatment (Figure 1c). In contrast, 0.5 Gy γ-irradiation or 150 μM HU did not induce significant p53 target gene expression (Figure 1c). Thus, activation of cell cycle arrest in wild-type MEFs correlates strongly with transactivation of target genes.

Although acute DNA damage treatment has been a useful tool to study the mechanisms of p53 activity in cell cycle arrest and senescence, the response to chronic, low-level DNA damage may represent a more accurate model for how p53 is induced in incipient tumor cells *in vivo*. Simply culturing primary MEFs *ex vivo* triggers a DNA damage response and replicative senescence after several passages, which is thought to result from oxidative stress that accumulates in the presence of 20% atmospheric oxygen. Accordingly, primary MEFs cultured in physiological oxygen levels (2–5%) do not activate a DNA damage response or undergo replicative senescence.[7,8] To ensure that the responses we measured were the result of the specified treatments and not damage triggered by growth at high oxygen tension and because the differences between culturing cells at atmospheric and physiological oxygen levels has not been analyzed systematically, we examined the cell cycle arrest responses to treatment with acute or low-dose DNA-damaging agents in cells cultured in 2% oxygen. Similarly to standard culture conditions, wild-type MEFs maintained in 2% oxygen and treated with 0.2 μg/ml doxorubicin or 12 Gy γ-irradiation underwent cell cycle arrest 24 h after treatment and displayed *p21* and *Mdm2* induction 2 h post treatment, while treatment with the lower dose of 0.5 Gy γ-irradiation or with 150 μM HU did not trigger cell cycle arrest or target gene induction (Figures 1d and f). Cells maintained in 2% oxygen also displayed p53 protein levels similar to cells at 20% oxygen after the various treatments, with a slight attenuation of p53 accumulation after doxorubicin treatment (Figure 1e). Analysis of Histone H2AX phosphorylation (γH2AX), a marker of DNA double-strand breaks, verified that DNA damage is present after both the acute and low-dose treatments, as numerous γH2AX foci were observed by immunofluorescence in wild-type MEFs 30 min after treatment with 0.2 μg/ml doxorubicin or 12 or 0.5 Gy γ-irradiation (Figure 1g).[28,29] These experiments thus establish a novel model system in which to study the chronic, low-dose DNA damage response in MEFs.

p19^{Arf} is dispensable for the response to acute DNA damage

To elaborate the role of p19^{Arf} in the DNA damage response, we first compared the responses of wild-type, *p53*-null and *p19^{Arf}*-null MEFs maintained in 2% oxygen to acute DNA damage (Figure 2a). 24 h after treatment with 0.2 μg/ml doxorubicin or 12 Gy γ-irradiation, wild-type MEFs underwent a clear proliferative arrest and ultimately senesced, as assessed by expression of senescence-associated β-galactosidase (SA-β-gal) 10 days post treatment (Figure 2b). As expected, *p53*-null MEFs were unable to mount a response to acute DNA damage, and showed only a mild decrease in proliferation following doxorubicin treatment. In contrast, MEFs deficient for *p19^{Arf}*, but retaining wild-type *p53,* underwent a proliferative arrest 24 h after doxorubicin or 12 Gy γ-irradiation treatment, followed by senescence 10 days later (Figure 2b). These data suggest that p19^{Arf} is dispensable for acute DNA damage responses.[18]

p19^{Arf} is required for the response to chronic, low-dose DNA damage

We next assessed whether p19^{Arf} might contribute to the cellular response to chronic, low-dose DNA-damaging agent treatment. Toward this end, we grew wild-type MEFs in 2% oxygen, treated them daily with 0.5 Gy γ-irradiation and measured proliferation at several time points (Figure 2c). After 10 days of treatment, wild-type MEFs underwent a complete cell cycle arrest accompanied by SA-β-gal positivity (Figure 2d). Importantly, untreated wild-type MEFs maintained in 2% oxygen showed only a slight reduction in proliferation over the 10-day time course (Figure 2e), indicating that the senescence response in treated wild-type MEFs is due to

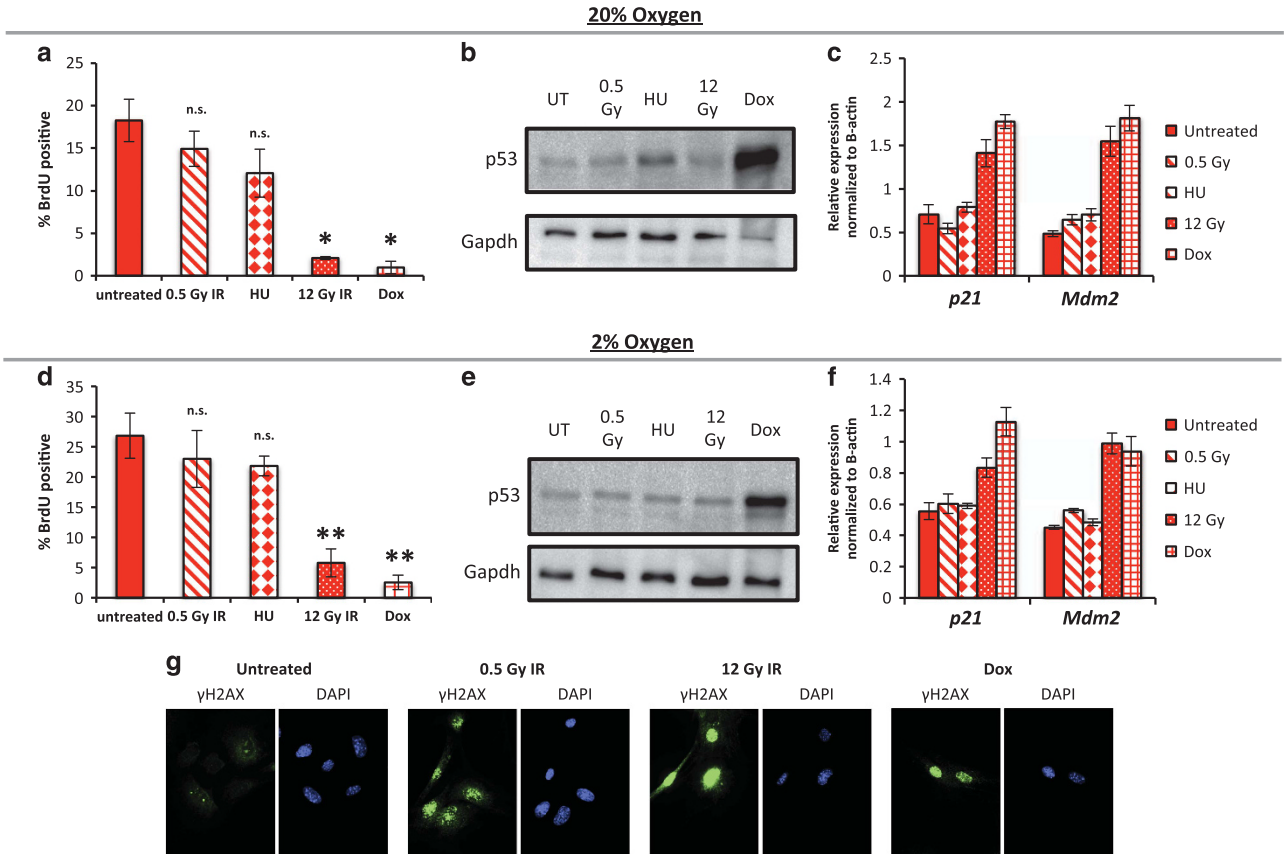

Figure 1. Establishing assays for acute and chronic DNA damage responses. (**a**) Effects of DNA-damaging agent treatment on cell cycle progression assessed by BrdU incorporation. Wild-type MEFs maintained in 20% (atmospheric) oxygen were treated with 0.2 μg/ml doxorubicin, 12 or 0.5 Gy of γ-irradiation (using a ^{137}Cs source), 150 μM hydroxyurea or left untreated. Twenty-four hours later, cells were pulsed with 3 μg/ml BrdU for 4 h, fixed in 4% paraformaldehyde and assessed for BrdU incorporation by immunofluorescence using an α-BrdU antibody (1:50, BD Biosciences, San Jose, CA, USA). Differences in BrdU incorporation between treated and untreated cells were analyzed by two-tailed *t*-test assuming unequal variance: 'n.s.', not significant ($P > 0.05$), *$P \leqslant 0.05$ and **$P \leqslant 0.01$. (**b**) Effects of DNA-damaging agent treatment on p53 protein levels. Western blotting for p53 (CM5 1:500, Vector Laboratories, Burlingame, CA, USA) was performed on wild-type MEFs maintained in 20% oxygen and treated with 12 or 0.5 Gy of γ-irradiation, 150 μM hydroxyurea or 0.2 μg/ml doxorubicin. Cells were harvested 8 h after treatment. Gylceraldehyde-3-phosphate dehydrogenase (Gapdh) serves as a loading control (1:15000, Fitzgerald, Acton, MA, USA). (**c**) Effects of DNA-damaging agent treatment on p53 target gene expression. RNA was harvested from wild-type MEFs maintained in 20% oxygen 2 h after treatment with 12 or 0.5 Gy of γ-irradiation, 150 μM hydroxyurea or 0.2 μg/ml doxorubicin, quantitative PCR with reverse transcription was used to analyze expression of *p21* and *Mdm2*. Expression levels are normalized to *β-actin*. The averages ± s.e.m. from three independent biological experiments are shown. (**d**) Effects of DNA-damaging agent treatment on cell cycle progression, as assessed by BrdU incorporation, in 2% oxygen. Wild-type MEFs maintained at 2% oxygen were treated with 0.2 μg/ml doxorubicin, 12 or 0.5 Gy of γ-irradiation (using a ^{137}Cs source), 150 μM hydroxyurea or left untreated. Twenty-four hours later, cells were pulsed with BrdU for 4 h, and assessed for BrdU incorporation by immunofluorescence as in **a**. Differences in BrdU incorporation between treated and untreated cells were analyzed by two-tailed *t*-test as in **a**. (**e**) Effects of DNA-damaging agent treatment on p53 protein levels in 2% oxygen. Western blotting for p53 was performed on wild-type MEFs maintained in 2% oxygen as in **b**. (**f**) Effects of DNA-damaging agent treatment on p53 target gene expression. RNA was harvested from wild-type MEFs maintained in 2% oxygen and analyzed as in **c**. (**g**) Measuring DNA damage in 2% oxygen. γH2AX immunostaining (Millipore, Darmstadt, Germany, 1:1000) on wild-type MEFs maintained at 2% oxygen and treated with 0.2 μg/ml doxorubicin, 12 Gy γ-irradiation, 0.5 Gy γ-irradiation or left untreated and then fixed in paraformaldehyde 30 min after treatment.

daily 0.5 Gy γ-irradiation and not simply the result of any residual culture shock in a 2% oxygen environment. In contrast to wild-type MEFs, *p53*-null cells showed no evidence for cell cycle arrest or senescence after 10 days of daily γ-irradiation. Interestingly, in response to chronic, low-dose irradiation, *p19^{Arf}*-deficient MEFs behaved similarly to *p53*-null cells, and neither mounted a cell cycle arrest response nor displayed senescence marker positivity after 10 days of daily 0.5 Gy γ-irradiation (Figure 2d). These findings indicate that p19^{Arf} is critical for the cell cycle arrest response to chronic, low-dose DNA damage.

To generalize our results using a mimic of the physiological stress of replication fork collapse, we extended our analysis to HU. Prolonged exposure to HU has been shown to promote cell cycle arrest,[30] and indeed, wild-type MEFs exposed to 150 μM HU exhibited a significant reduction in BrdU incorporation after 7 days and senescence after 10 days (Figure 2f). In the same time period, *p53*-null cells did not mount a significant cell cycle arrest response. Similarly, the ability of *p19^{Arf}*-deficient MEFs to undergo cell cycle arrest and senescence in response to chronic HU treatment was largely compromised (Figure 2f), confirming a role for p19^{Arf} in the response to chronic, low-dose DNA damage.

The role of p53 in the chronic DNA damage response in *p19^{Arf}* null cells

The classical role for p19^{Arf} downstream of oncogene activation is stabilization of p53 via inhibition of the negative regulator Mdm2. The same molecular mechanism could underlie the role of p19^{Arf}

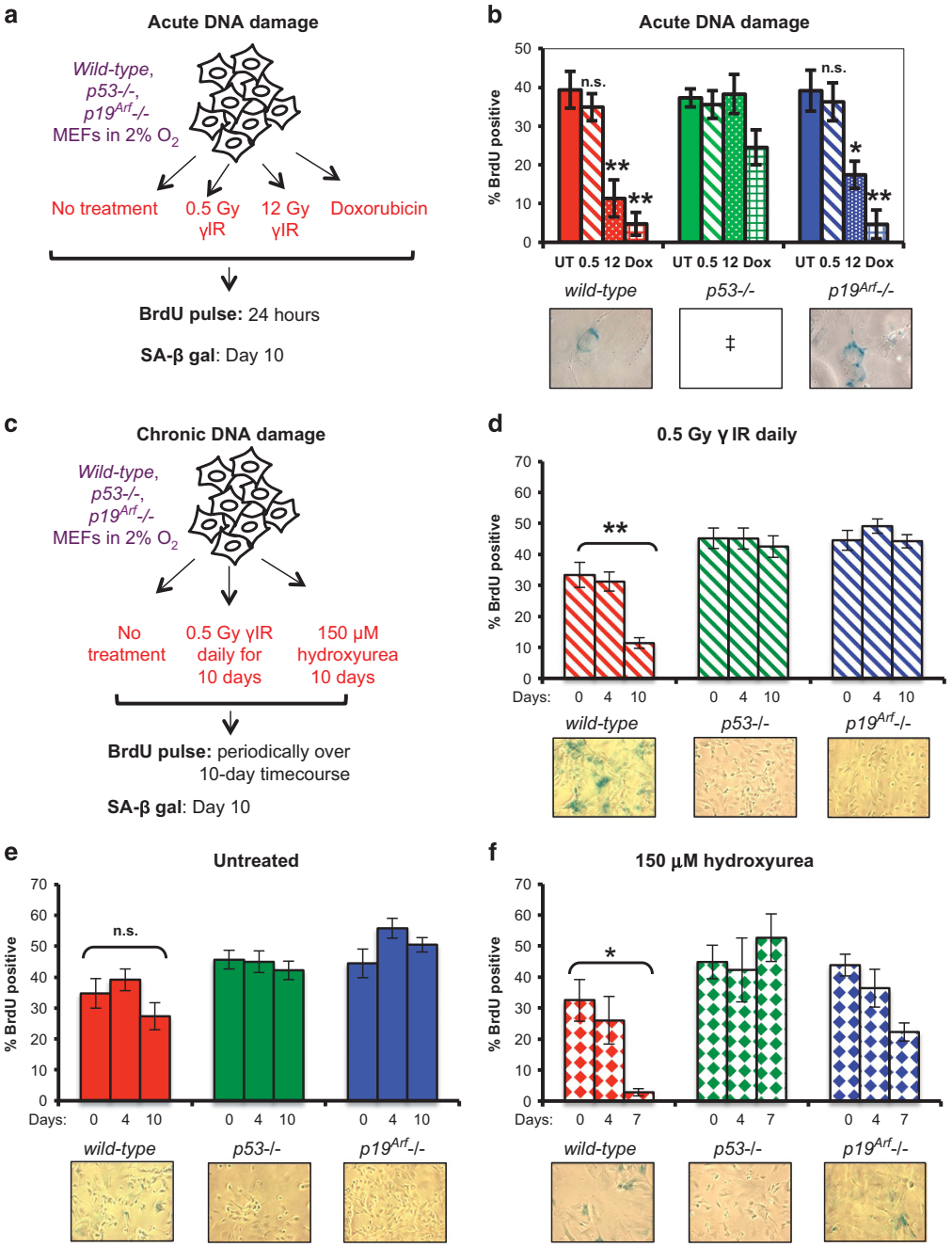

Figure 2. p19^{Arf} is dispensable for the response to acute DNA damage but is required for the response to chronic DNA damage. (**a**, **b**) Assessing acute DNA damage responses. Wild-type, *p53*-null and *p19^{Arf}*-null MEFs maintained in 2% O_2 were treated with 0.2 μg/ml doxorubicin, 12 or 0.5 Gy of γ-irradiation or left untreated. Twenty-four hours after treatment, cells were pulsed with BrdU and assessed for BrdU incorporation by immunofluorescence as in Figure 1a. Ten days after treatment, cells were fixed in 2% formaldehyde/0.2% glutaraldehyde and incubated with X-gal at pH 6.0 for 48 h as described.[44] Shown are representative photomicrographs of SA-β gal-stained wild-type and *p19^{Arf}*-null MEFs treated with doxorubicin. '‡' Indicates that photomicrographs of *p53*-null MEFs treated with doxorubicin could not be obtained at this time point because the cells had died because of a lack of cell cycle arrest. Differences in BrdU incorporation between treated and untreated cells of each genotype were analyzed by two-tailed *t*-test as in Figure 1a; n.s., not significant ($P > 0.05$), *$P \leqslant 0.05$, **$P \leqslant 0.01$. Comparisons between genotypes were based on the ratio of treated to untreated cells as follows: wild type (0.5 Gy/UT) vs *p19^{Arf}* (0.5 Gy/UT), $P = 0.97$; wild type (12 Gy/UT) vs *p19^{Arf}* (12 Gy/UT), $P = 0.24$; wild type (Dox/UT) vs *p19^{Arf}* (Dox/UT) $P = 0.56$. (**c–f**) Assessing chronic DNA damage responses. (upper) Wild-type, *p53*-null and *p19^{Arf}*-null MEFs maintained at 2% O_2 were treated with 0.5 Gy of γ-irradiation daily (**d**), were left untreated (**e**) or were treated with 150 μM hydroxyurea (HU) (**f**). Cells were pulsed with BrdU at the indicated time points and assessed for BrdU incorporation by immunofluorescence as in Figure 1a. The graphs represent the mean of 3–6 independent experiments and the error bars represent the s.e.m. Differences in BrdU incorporation between days 0 and 10 cells of different genotypes were analyzed by two-tailed *t*-test as in **b**. Comparisons between genotypes were based on the ratio of day 10 (or day 7 for HU) to day 0 cells as follows: wild-type UT (day 10/day 0) vs *p19^{Arf}* UT (day 10/day 0), $P = 0.13$; wild-type 0.5 Gy (day 10/day 0) vs *p19^{Arf}* 0.5 Gy (day 10/day 0), $P = 0.0045$; wild-type HU (day 7/day 0) vs *p19^{Arf}*HU (day 7/day 0), $P = 0.028$. (lower) Representative images of MEFs of each genotype stained for SA-β gal positivity as in **b** on day 10.

in the response to chronic, low-dose DNA damage. Alternatively, the chronic DNA damage response could occur through a mechanistically distinct pathway, in which the role for p19Arf is independent of p53. To begin to distinguish these possibilities, we assessed p53 protein stabilization in wild-type and *p19Arf* −/− MEFs after 4 days of daily 0.5 Gy γ-irradiation treatment, before we observe an arrest response. Interestingly, while basal p53 levels are notably lower in *p19Arf* −/− MEFs than in wild-type MEFs, as observed previously,[18] after 4 days of chronic DNA-damaging agent treatment, p53 levels in *p19Arf* −/− MEFs approach levels in wild-type MEFs (Figure 3a). These data suggest that the inability of *p19Arf* −/− MEFs to senesce in response to chronic DNA damage is not due to a complete lack of p53 stabilization. To further probe the p53-dependence of the role of p19Arf in the chronic DNA damage response, we examined the expression of p53 target genes after 4 days of daily 0.5 Gy γ-irradiation treatments. First, we found that wild-type MEFs maintained at 2% O_2 displayed increased expression of p53 target genes upon chronic DNA-damaging agent treatment (Figure 3b). Second, and consistent with the observed increase in p53 protein stability upon chronic DNA damage exposure, we also observed increased expression of *p21*, *Mdm2* and *Ccng1* in *p19Arf*-null MEFs treated with chronic damage relative to untreated *p19Arf*-deficient cells, while these transcripts were not induced in *p53*-null MEFs (Figure 3b). Taken together, these data demonstrate that the p53 pathway is quite functional in *p19Arf*-null MEFs subjected to a chronic, low-dose DNA-damaging agent treatment, suggesting that complete disruption of the p19Arf-p53 axis does not underlie the failure to undergo senescence that we observed in *p19Arf*-null cells. However, the extent of p53 accumulation and p53 target gene activation in *p19Arf*-null cells is slightly reduced relative to wild-type cells, suggesting that the ability of p19Arf to 'fine-tune' p53 responses and stimulate full p53 induction contributes to the chronic DNA damage response.

We reasoned that if the p19Arf-p53 axis underlies the role of p19Arf in the response to chronic DNA damage, we could activate p53 downstream of p19Arf and promote cell cycle arrest in *p19Arf* −/− cells. Conversely, if p53 activation in *p19Arf* −/− cells fails to promote cell cycle arrest in response to chronic damage, then some other p53-independent mechanism must be required for p19Arf function in this context. To distinguish these models, we used the p53 activator Nutlin3a. As an inhibitor of the p53-Mdm2 interaction, Nutlin3a liberates p53 to trigger target gene activation and cell cycle arrest.[31] We treated MEFs with 1 μM Nutlin3a, a dose that we found could promote cell cycle arrest or senescence in wild-type MEFs, but only when combined with chronic γ-irradiation, indicating that the arrest depends on the chronic DNA-damaging agent treatment. In contrast, p53-null MEFs did not mount an arrest or senescence response, indicating that this cell cycle arrest response is p53-dependent (Figures 4a and b). Although wild-type MEFs treated with the vehicle control (dimethyl sulfoxide) and subjected to daily 0.5 Gy γ-irradiation treatments underwent a statistically significant arrest and senesced after 10 days of treatment, combination treatment with both 1 μM Nutlin3a and 0.5 Gy γ-irradiation daily triggered a more

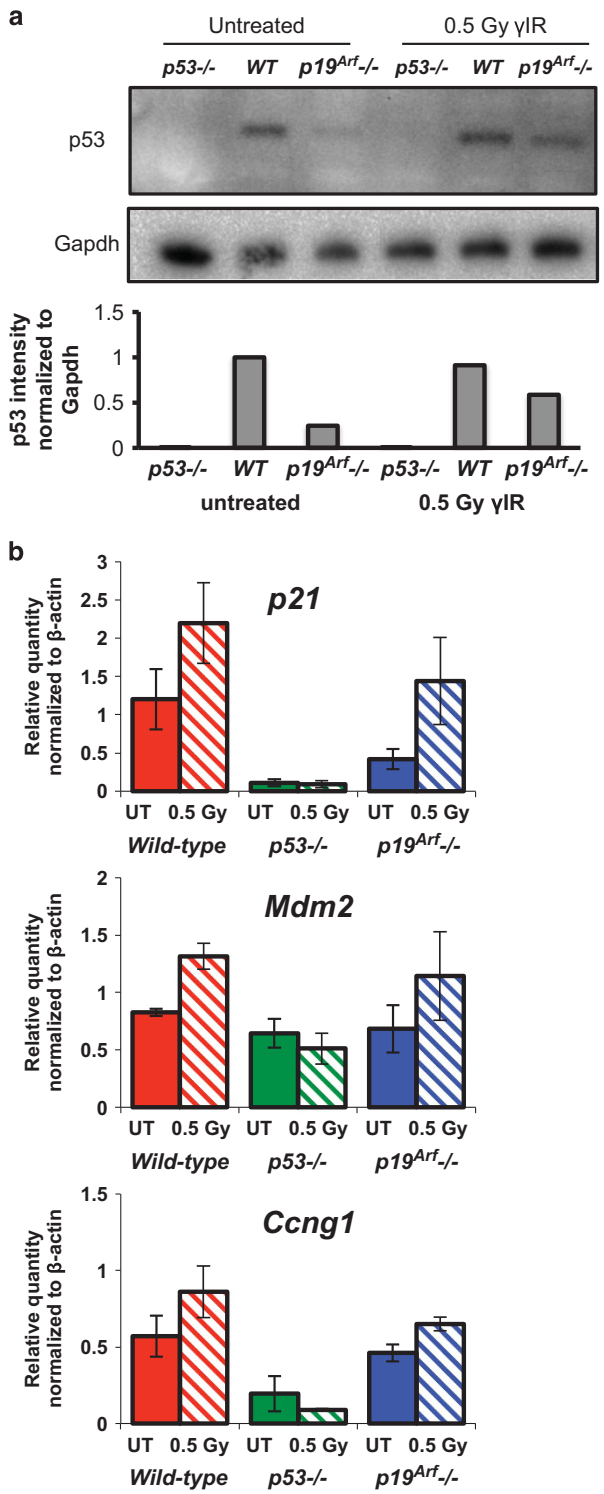

Figure 3. p53 protein stabilization and target gene induction in the chronic DNA damage response. (**a**) p53 levels upon chronic DNA damage. Wild-type, *p53*-null and *p19Arf*-null MEFs maintained at 2% O_2 were treated with 0.5 Gy of γ-irradiation daily or left untreated. (top) Protein extracts were prepared after 4 days of treatment and analyzed by immunoblot for p53 (CM5, 1:500, Vector Laboratories). Gapdh (1:15 000, Fitzgerald) serves as the loading control. (bottom) The chemiluminescent blots were imaged using the ChemiDoc XRS + (Bio-Rad, Hercules, CA, USA) system with ImageLab software (Bio-Rad) to select the appropriate exposure for quantitation. Image J software (National Institutes of Health, Bethesda, MD, USA) was used to quantify band intensities, and relative intensities were determined by normalization of p53 to GAPDH. A representative experiment from three independent trials is shown. (**b**) p53 target gene expression upon chronic DNA damage. Wild-type, *p53*-null, and *p19Arf*-null MEFs maintained at 2% O_2 were treated with 0.5 Gy of γ-irradiation daily or left untreated. RNA was prepared after 4 days of treatment and analyzed for *p21, Mdm2* or *Ccng1* expression by quantitative PCR with reverse transcription. Expression levels are normalized to *β-actin*. The averages ± s.e.m. from three independent biological experiments are shown.

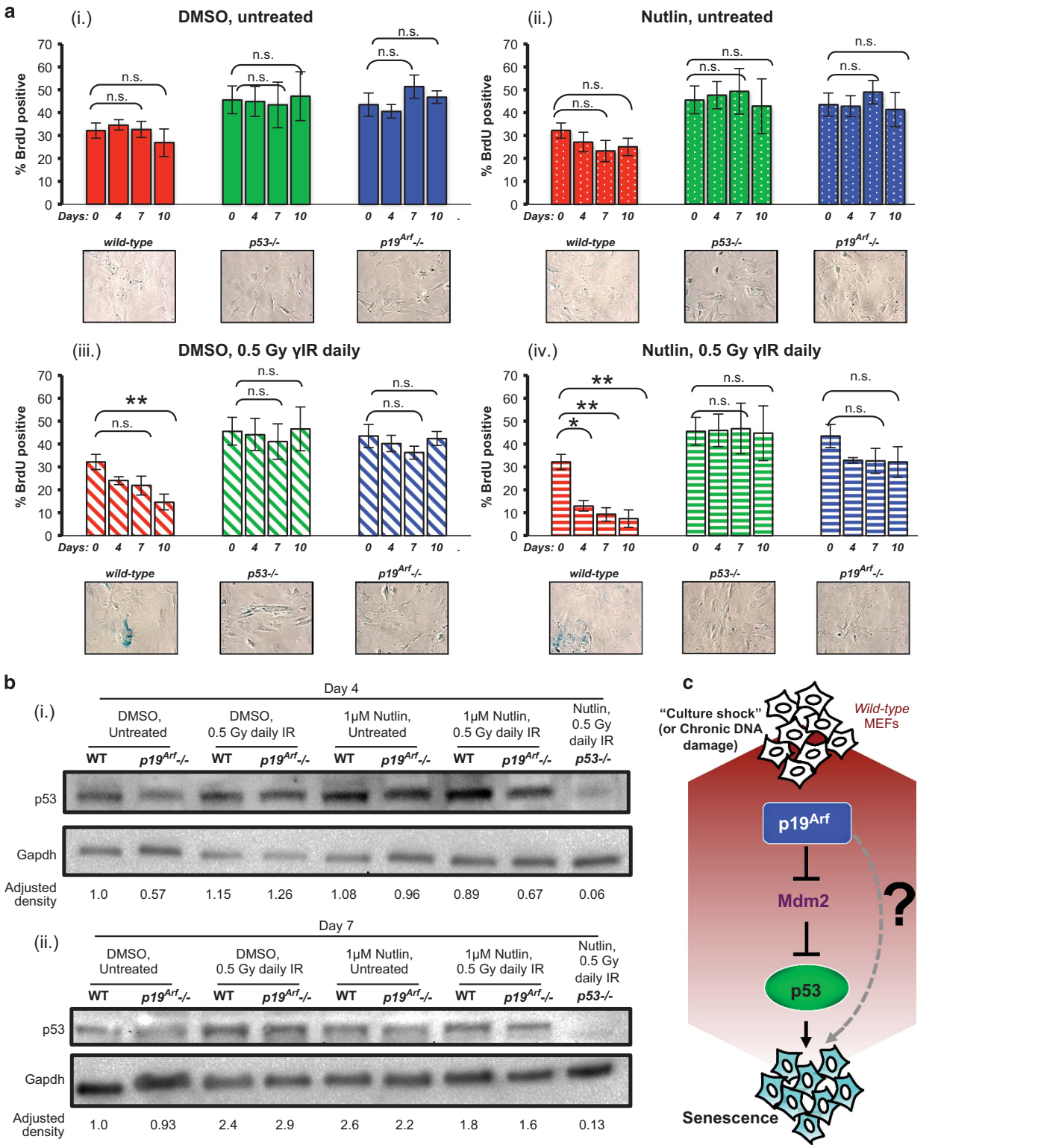

Figure 4. Activation of p53 with Nutlin3a fails to restore cell cycle arrest and senescence in *p19*^{Arf}-null MEFs. (**a**) Effects of chronic DNA damage and Nutlin3a on cell cycle progression. (upper) Cells were maintained in 2% oxygen and treated with 1 μM Nutlin3a or dimethyl sulfoxide (vehicle) and exposed to 0.5 Gy of γ-irradiation daily or left untreated. BrdU incorporation was assessed on days 0, 4, 7 and 10 as described in Figure 1a. The graphs show the averages ± s.e.m. of three time courses, and *P*-values were calculated with a two-tailed *t*-test assuming unequal variance. (lower) Representative images of MEFs from each genotype stained for SA-β gal positivity as in Figure 2b on day 10. Higher magnification images of additional fields of SA-β gal-stained cells are shown in Supplementary Figure 1. (**b**) Effects of chronic DNA damage and Nutlin3a on p53 protein levels. Cells were treated as above, and protein extracts were prepared after 4 days (i) or 7 days (ii) of treatment and analyzed by immunoblot for p53 as in Figure 3a. Gapdh serves as the loading control. A representative experiment from three independent trials is shown. (**c**) Our findings suggest an underlying basis for senescence of mouse fibroblasts in culture. In response to chronic, low-dose DNA damage, as occurs during passage in culture, p19^{Arf} acts both through activation of p53 and potentially through a p53-independent pathway, that remains to be elucidated (indicated by '?').

potent cell cycle arrest in wild-type MEFs. This cell cycle arrest, in contrast to that triggered by daily 0.5 Gy γ-irradiation alone, was statistically significant by day 4, and also promoted senescenceby day 10 (Figure 4a, Supplementary Figure 1). Interestingly, *p19^{Arf}*-deficient cells did not display appreciable cell cycle arrest or senescence when treated with both 1 μM Nutlin3a and daily low-dose irradiation (Figure 4a, Supplementary Figure 1). p53 protein levels were not substantially different between the wild-type and *p19^{Arf}*−/− samples, suggesting that gross variations in p53 stabilization do not appear to underlie the differences between the observed phenotypes in these two genotypes (Figure 4b).

Collectively, our observations provide genetic evidence that p19^{Arf} is dispensable for the acute DNA damage response but has a critical role in the response to chronic, low-level DNA damage. Notably, we do not observe significant induction of *p19^{Arf}*upon exposure of cells to chronic DNA damage, either at the mRNA level or at the protein level, as assessed by analysis of p19^{Arf} localization to nucleoli, where it is stable and active[32] (Supplementary Figure 2). Therefore, although p19^{Arf} is important for the response to chronic DNA damage, it is not clearly upregulated by such signals. The notion that p19^{Arf} is important for the chronic DNA damage response is supported by other studies. For example, one study reported a partial impairment in the DNA damage response in p19^{Arf}-deficient MEFs.[18] Moreover, Arf depletion in Brca2-deficient MEFs or human cells rescued senescence[33] and deletion of the Ink4a/Arf locus (both p19^{Arf} and p16^{Ink4a}) rescued premature senescence in cells with a hypomorphic ATR mutation, which induces replication fork collapse and DNA damage.[34] In addition, although p19^{Arf} acts at least in part by promoting the efficient activation of p53, our experiments using Nutlin3a in combination with chronic low-dose irradiation suggest the possibility of a p53-independent role for p19^{Arf} in the response to chronic DNA damage (Figure 4c). Numerous activities beyond p53 regulation have been described for p19^{Arf}, including regulating superoxide production,[35] ribosomal biogenesis[36] and sumoylation of interacting proteins.[37] Analysis of such Arf functions in the responses to chronic, low-level DNA damage will be very interesting avenues for future investigation.

Here, we leverage the widely used primary MEF model to better understand the contribution of p19^{Arf} to senescence and tumor suppression in response to chronic, low-dose DNA damage. Evidence for an activated a DNA damage response has been found in developing human tumors, leading to a model in which oncogenic signaling induces DNA replication stress and double-strand breaks, engendering a DNA damage response that triggers p53 activation.[20,21,25] DNA damage signals are complemented by oncogene-mediated activation of Arf to promote senescence and suppress tumorigenesis, through p53 activation and/or p53-independent functions of Arf.[26] Our studies provide additional insight into how incipient tumor cells can activate the p19^{Arf}-p53 suppressor axis as a result of the chronic DNA damage they sustain, illuminating a link between the activated DNA damage response observed in nascent tumors and the functional requirement for p19^{Arf} in tumor suppression. Notably, several studies using mouse models have suggested that the p53 acute DNA damage response is dispensable for tumor suppression,[12,13,38–41] a notion seemingly at odds with the presence of activated components of the DNA damage response cascade seen in human tumors. Our results resolve this discrepancy by suggesting that the response to chronic, low-level DNA damage is a p19^{Arf}-dependent mechanism that is distinct from the response to acute DNA damage and may be particularly important for p53-mediated tumor suppression. Continued investigation into the complex interplay between the DNA damage response, Arf, and tumor suppression—an ever-evolving area[42,43]—will ultimately help decipher key tumor suppressor pathways.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)